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Isoflavone Profile and Biological Activity of Soy Bread

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The present study examines the ability of isoflavone extracts from whole soy bread and two soy bread fractions, crumb and crust, to modulate the proliferation of human prostate cancer PC-3 cells. Total isoflavone content in the two fractions of soy bread were similar (3.17 μmol/g dry basis). However, their conjugate patterns were altered. Both fractions of soy bread contained a similar level of isoflavone aglycones (∼24%). Low concentrations of soy bread extracts increased PC-3 cell proliferation as much as 47% compared to untreated control. This proliferative effect in cell growth was reduced at higher extract concentration. Soy bread crust extract (10 mg/mL) reduced PC-3 cell proliferation by 15% compared to untreated control. Interestingly, wheat bread extracts increased cell proliferation at all concentrations tested. Although extracts from both breads possessed biological activity, only soy bread crust extract reduced PC-3 cell proliferation. This observation may be related to the presence of soy in this bread.

KEYWORDS: Soy bread; isoflavones; cancer cell proliferation

INTRODUCTION

Soy contains proteins and other biologically active components (isoflavones) that may be effective in reducing the risk of coronary heart diseases and several cancers (*1*−*6*). For example, higher consumption of soy foods in Asia is associated with lower risk of prostate cancer compared to the U. S. (*7*−*11*). Incorporating soy into a staple food (i.e., bread) may be a feasible means of increasing daily soy intake in people following typical Western diets (*12*−*14*). We developed a highly acceptable soy-enriched bread ("soy bread") that contains sufficient soy proteins per serving to meet the FDA-approved health claim on coronary heart disease (*15*, *16*).

Isoflavones are plant-derived compounds with structural similarity to estrogen (*17*−*20*). Genistein, daidzein, and glycitein are the three most prevalent isoflavones in soybeans. The predominant (∼95%) isoflavones in unprocessed soybeans are the malonylglucosides (*21*, *22*). Processing, i.e., heat treatment, is responsible, in part, for the changes in isoflavone content and composition in processed soy foods (*23*−*25*). Recent studies suggested the chemical forms and abundance of isoflavones in soy foods have a significant impact on their bioavailability and biological effects (*26*−*30*).

Isoflavones have been shown to inhibit tumor growth and development in several in vitro and in vivo models (*11*, *18*, *31*, *32*). However, relatively little data are available regarding the biological activity of isoflavones delivered in a food matrix or the effect of processing on these bioactive compounds. Previous studies have shown that the biological activity of isoflavones from whole soy foods may depend on the processing conditions, the type of soy foods, and interaction with other constituents of the diet (*33*, *34*). The present study examines the isoflavone content and composition in soy bread, and the effects of soy bread extracts on the proliferation of an invasive androgen independent metastatic human prostate cancer cell line (PC-3).

MATERIALS AND METHODS

The experimental plan of this study is shown in **Figure 1**.

Materials. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and isoflavone pure standard compounds were purchased from Sigma (St. Louis, MO) and LC labs (Wolburn, MA). All organic solvents and chemical reagents were HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ). PC-3 cells were obtained from ATCC (Manassas, VA). Tissue culture supplies were from Gibco (Grand Island, NY). Soy flour was obtained from Archer Daniel Midland Co. (Decatur, IL). Soy milk powder was obtained from DevanSoy Farms (Carroll, Iowa).

Bread Preparation. The main ingredients in bread making are presented in **Table 1**. Wheat bread was made following a conventional wheat bread formula. Soy bread was prepared using a patent-pending process that involves replacing 49% of wheat flour (General Mills, Minneapolis, MI) in wheat bread formula with soy flour and soy milk powder. The bread ingredients were combined and mixed in a 5-quart Kitchen Aid Mixer (KitchenAid Portable Appliance, St. Joseph, MI) to form dough. The dough was proofed at 50 °C for 1 h in an oven (Blue M Electric Company, Blue Island, ILL) and baked at 165 °C for 50 min. The proximate composition of wheat bread and soy bread is listed in **Table 2**. After the loaves were cooled, the crumb and crust were separated from breads based on color difference (darker = crust, lighter = crumb). All the bread samples were stored at −20 °C for subsequent HPLC analysis and MTT proliferation assay.

Extraction of Isoflavones from Bread Samples. Three samples were obtained from each bread for isoflavone extraction. Samples from soy bread were soy bread crumb (fraction 1S), soy bread crust (fraction

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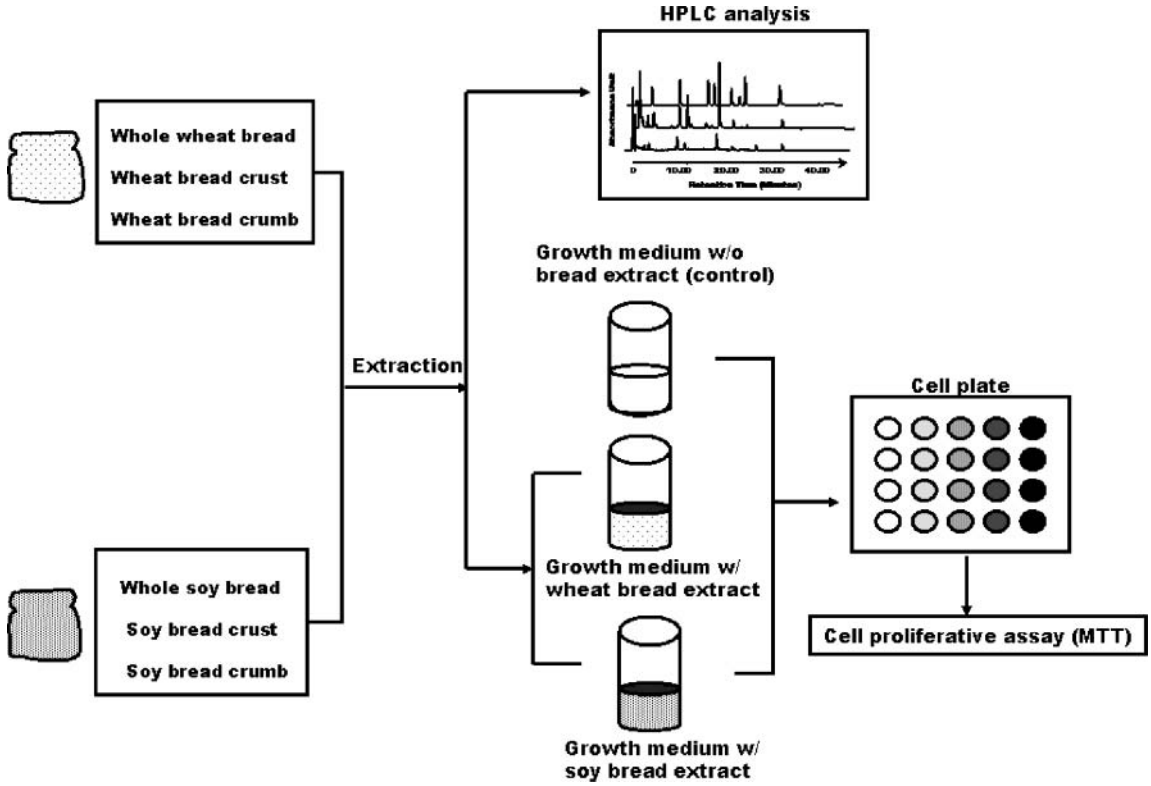

Figure 1. Experimental plan for HPLC analysis and MTT assay.

Table 1. Ingredients Used in Making Wheat and Soy Bread[a]

ingredient	soy bread % (w/w)	wheat bread % (w/w)
H_2O	45.3	37.7
soy milk powder	6.6	0
soy flour	19.9	0
wheat flour	17.5	54.3
pure gluten	2.3	0
dough conditioner	0.2	0
sugar	4.5	4.0
yeast	1.0	0.9
salt	0.9	1.0
shortening	1.7	2.1

[a] Ingredients were added as is (wet basis).

Table 2. Proximate Chemical Composition of Wheat Bread and Soy Bread

composition (%)	wheat bread	soy bread crumb	soy bread crust
moisture	37	44	16
carbohydrate[a]	81	40	40
fat[a]	2	2	2
protein[a]	14	34	34

[a] Expressed on a dry basis. Values are averages of three repetitions.

2S), and whole soy bread (fraction 3S) which contained both crumb and crust. Samples from wheat bread were wheat bread crumb (fraction 1W), wheat bread crust (fraction 2W), and whole wheat bread (fraction 3W) which contained both crumb and crust. Bread sample (0.5 g) was ground to fine paste and mixed with 0.1 N HCl (2 mL), acetonitrile (10 mL), and water (3 mL). The mixture was shaken with a multi-wrist shaker (Lab-line Instrument Inc, Melrose Park, IL) at speed 9 at room temperature for 2 h and centrifuged at 430*g* for 30 min in a centrifuge (IEC HN−SII, Damon/IEC Division, Needhamhts, MA). The supernatant was collected and dried under nitrogen with an evaporating unit (Pierce model 18780, Rockford, IL). Dried residue was redissolved in 100% methanol of equal amount and stored at −20 °C for subsequent HPLC analysis.

HPLC Analysis. A Waters 2695 separation module (Milford, MA) fitted with a Waters 2996 photodiode Array detector ("PDA") was used to quantify the isoflavone content. Separation of isoflavone was achieved using a Waters Nova-Pak C18 reversed-phase column (3.9 × 150 mm; i.d. 4 μm, 60 Å pore size). The PDA detection monitored from a wavelength range of 210−400 nm, and the eluting compounds were detected at 260 nm. The mobile phase consisted of 1.0% acetic acid in water (v/v) (solvent A) and 100% acetonitrile (solvent B). The elution condition was as follows: 0−5 min 15%B, 5−36 min 15−29%B, 36−44 min 29−35%B, 44−45 min 35−15%B, reequilibrate at 15%B for 5 min for next run. Flow rate was at 0.6 mL/min. Injection volume was 10 μL.

Identification and Quantification of Isoflavones. The isoflavones in bread samples were identified by comparison of retention time and UV absorbance patterns with pure isoflavone compounds. Identification results were confirmed by enzymatic hydrolysis with deconjugating enzyme, β-glucosidase. Concentration of each isoflavone was determined by the HPLC peak area and corresponding standard curve.

MTT Proliferation Assay. The MTT assay utilizes 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is reduced to an insoluble dye by mitrochondrial enzymes. This reduction is associated with the metabolic activity of living cells, and can be quantified through spectrophotometry using a 96-well plate reader.

Aliquots of the solvent extract of whole soy bread, its crust and crumb, whole wheat bread, its crust and crumb, were dried under vacuum (Savant Instruments Inc., Farmingdale, NY) at room temperature. Dried extracts were then weighed and redissolved into RPMI 1640 (+ 10% FBS) at a concentration range of 0−10 mg of extract/mL of growth medium. The medium containing extract was sterile filtered using a 0.2-micron filter and refrigerated until use, usually within 24 h.

PC-3 prostate cancer cells were plated at a density of 1×10^5 cells/well in a 96-well tissue culture plate. The cells were incubated for 24 h at 37 °C with 150 μL of RPMI 1640/well. After 24 h, the growth medium was removed and 150 μL of RPMI 1640 containing serial 2-fold dilutions of bread extracts was added to each well. The plates were then incubated for 48 h. Cells receiving no bread extracts served as control.

Table 3. Isoflavone Content (nmol/g dry basis)[a] and Composition of Soy Bread (%)

isoflavone	soy bread crumb (fraction 1S)	%	soy bread crust (fraction 2S)	%	whole soy bread (fraction 3S)	%
daidzin	253.7 ± 20.3		381.9 ± 22.9		278.3 ± 15.5	
genistin	782.6 ± 7.8		1000.0 ± 10.0		806.1 ± 5.7	
β-glucosides	**1036.3**	**32.7**	**1381.0**	**43.6**	**1084.4**	**34.2**
6-*O*″-malonyldaidzin	315.0 ± 1.0		73.2 ± 0.7		272.6 ± 0.8	
6-*O*″-malonylgenistin	789.6 ± 7.9		160.7 ± 0.0		763.9 ± 4.0	
6-*O*″-malonylglycitin	35.2 ± 0.4		36.7 ± 0.7		35.1 ± 0.3	
6-*O*″-malonylglucosides	**1139.8**	**36.0**	**270.6**	**8.6**	**1071.6**	**33.8**
6-*O*″-acetyldaidzin	57.9 ± 1.2		178.7 ± 1.8		62.1 ± 1.2	
6-*O*″-acetylgenistin	95.2 ± 2.9		488.1 ± 9.8		109.2 ± 2.4	
6-*O*″-acetylglycitin	70.4 ± 4.2		68.3 ± 3.4		71.6 ± 2.3	
6-*O*″-acetylglucosides	**223.5**	**7.0**	**735.1**	**23.2**	**242.9**	**7.7**
daidzein	306.7 ± 3.1		315.0 ± 6.3		308.9 ± 5.5	
genstein	448.9 ± 9.0		447.0 ± 0.0		448.2 ± 3.4	
glycitein	14.3 ± 0.9		15.6 ± 0.3		14.9 ± 1.0	
aglycones	**769.9**	**24.3**	**777.6**	**24.6**	**772.0**	**24.3**
total	**3169.5**	**100**	**3164.3**	**100**	**3170.9**	

[a] Values are means ± SD of three independent determinations.

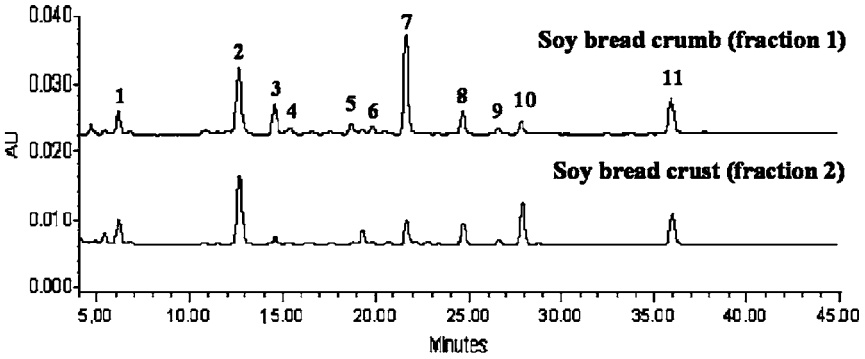

Figure 2. Typical reversed-phase HPLC chromatograms of extracts from soy bread crumb (fraction 1S) and crust (fraction 2S). Each extract was from 0.5 g of material on a wet basis and monitored at 260 nm. Peak identification for representative chromatograms are as follows: (1) daidzin, (2) genistin, (3) malonyldaidzin, (4) malonylgenistin, (5) acetyldaidzin, (6) acetylglycitin, (7) malonylgenistin, (8) daidzein, (9) glycitein, (10) acetylgenistin, (11) genistein.

After incubation, 10 μL of MTT solution (5 mg MTT/mL saline) was added to each well and allowed to incubate for at least 3 h. Growth medium was removed and 150 μL of 0.04 M HCl in isoproponal was added to each well. Plates were incubated for an additional 30 min, and the absorbance was read at 620 nm using a microplate reader (Multiskan MCC/340, Fisher Scientific, Atlanta, GA).

Statistical Analysis. Each sample was analyzed in triplicate. All data were expressed as means ± SD unless otherwise indicated. Statistical analysis was done by using the SAS software (SAS Inc, Cary, NC). Analyses of variance (ANOVA) using the general linear models (GLM) were conducted. Difference between the sample means were analyzed by Fisher's least significance (LSD) test at $\alpha = 0.05$. ANOVA and Tukey's post hoc tests were performed on data collected from MTT assays ($p < 0.05$).

RESULTS

Isoflavone Content and Composition of Soy Bread. The isoflavone content and composition of whole soy bread (fraction 3S) and its crumb and crust (fraction 1S and 2S) are shown in **Table 3**. Eleven isoflavone isomers except glycitin were clearly separated, identified, and quantified (**Figure 2**). Total isoflavone content was 3.17 μmol/g of dry weight in whole soy bread (fraction 3S) and soy bread crumb (fraction 2S), and 3.16 μmol/g in soy bread crust (fraction 3S). Whole soy bread (fraction 3S) and soy bread crumb (fraction 1S) were similar in isoflavone composition. However, soy bread crumb (fraction 1S) and crust (fraction 2S) differed in isoflavone composition except for their aglycone levels (24.3 and 24.6%). Soy bread crust contained a higher level of β-glucosides (43.6%) and 6-*O*″-acetylglucosides (23.2%) and a much lower level of 6-*O*″-malonylglucosides (8.6%) of isoflavones compare to soy bread crumb (32.7, 7.0, and 36.0%, respectively).

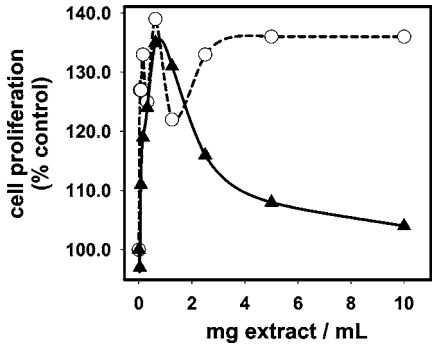

Figure 3. Effects of whole soy bread (fraction 3S) and whole wheat bread (fraction 3W) extracts on the proliferation of PC-3 prostate cancer cells. Data points ($n = 8$) represent cell proliferation as a percentage of untreated controls (100%). Whole soy bread extract: triangle; whole wheat bread extract: open circle.

Biological Activity of Bread Extracts. Low concentrations (0.03−1.25 mg/mL) of whole soy bread extract (fraction 3S) increased the proliferation of PC-3 cells by as much as 39% versus control cells receiving no extract (**Figure 3**). This increase in proliferation was reduced at higher concentrations (1.25−10.00 mg/mL) of extract. No decrease in cell proliferation was

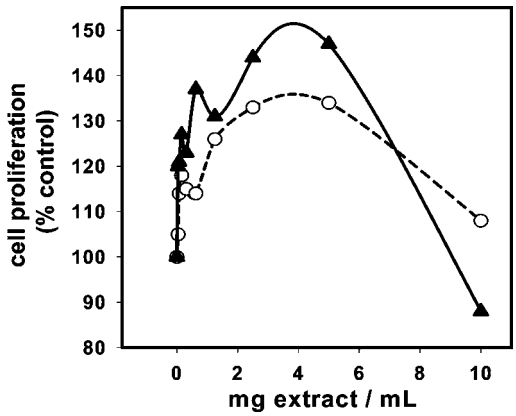

Figure 4. Effects of soy bread crumb (fraction 1S) and crust (fraction 2S) extracts on the proliferation of PC-3 prostate cancer cells. Data points ($n = 8$) represent cell proliferation as a percentage of untreated controls (100%). Soy bread crumb extract: open circle; soy bread crust extract: triangle.

observed at any concentration of whole soy bread extract tested. Soy bread crumb and crust extracts (fraction 1S and 2S) increased PC-3 cell proliferation by as much as 47 and 34%, respectively (**Figure 4**). No decrease in cell proliferation was observed at any concentration of soy bread crumb extract (fraction 1S) tested (maximum 10 mg/mL). Conversely, soy bread crust extract (fraction 2S, 10 mg/mL) reduced PC-3 cell proliferation by 15% versus control cells receiving no extract ($p < 0.05$). PC-3 cells treated with extracts from all wheat bread samples (fraction 1W, 2W, and 3W) showed an increase in proliferation over all concentrations tested.

DISCUSSION

Isoflavone Content and Composition of Soy Bread. A similar concentration of isoflavones was found in both fractions (crust and crumb) of soy bread after moisture normalization. However, their isoflavone profiles differed (**Table 3**). The difference in exposure temperature (crumb ~ 100 °C, crust ~ 165 °C) and moisture content (crumb ~ 44%, crust ~ 16%) may be among the factors contributing to different isoflavone profiles between these two fractions. Differences in heat exposure of crust and crumb did not affect the concentration of their aglycones (~24%). However, changes in 6-*O*″-acetylglucosides (+16.2%), β-glycosides (+10.9%), and 6-*O*″-malonylglucosides (−27.4%) were observed for crust compared to crumb. These findings agree with Coward et al.'s observation (1998) that the isoflavones glucoside conjugates are easily altered during processing and cooking. Particularly, dry heat results in the formation of acetylglucosides, and fermentation causes a loss of the glucosides to produce the aglycones. Our results along with Singletary et al.'s observation that extrusion did not cause a de-esterification of the glycosides to yield aglycones suggested that the thermal condition during baking may not be directly responsible for aglycone formation.

Biological Activity of Bread Extracts. There are conflicting data regarding the anticancer properties of soy isoflavones. Soy isoflavones have been shown to both increase and decrease the in vitro and in vivo proliferation of cancer cells (*35*−*37*). The majority of studies examining the biological effects of isoflavones utilize either purified compounds or soy protein isolates. The present study was designed to examine the biological effects of soy isoflavones delivered in a whole food system (bread).

The concentration of isoflavones in the extracts (0−10 mg/mL) applied to the PC-3 cells from whole soy bread (fraction 3S) were approximately 0−15 μM genistein and 0−10 μM daidzein, from soy bread crust (fraction 2S) approximately 0−33.8 μM genistein and 0−23.7 μM daidzein, and from soy bread crumb (fraction 1S) approximately 0−26.7 μM genistein and 0−18.2 μM daidzein. Isoflavones in wheat bread (fraction 1W, 2W, and 3W) were undetectable.

The results of our study show an increase in PC-3 prostate cancer cell proliferation upon exposure to low concentrations (0.03−1.25 mg/mL) of all bread extracts. These data are in agreement with others (*35*, *38*, *39*) demonstrating that low doses of soy isoflavones can increase cancer cell proliferation. This mechanism has been attributed to estrogenic activity of isoflavones (*40*). Interestingly, a similar increase in the proliferation of PC-3 cells was observed with exposure to whole wheat bread (fraction 3W) extract in **Figure 3**, indicating the mechanism for increased cell proliferation may be unrelated to isoflavone content and/or estrogenic activity.

A decrease in PC-3 cell proliferation was only observed upon treatment with soy bread crust (fraction 2S) extract in **Figure 4**. Although the total isoflavone in soy bread crust and crumb (fraction 1S, fraction 2S) extracts were similar, their isoflavone profile (**Table 3**) and physiochemical properties were different after processing. The bread crust contained less moisture (16%) than the bread crumb (44%), and was also the major site for Maillard reactions. Recently, a potent antioxidant derived from the Maillard reaction was found in bread crust (*41*). This antioxidant was shown to induce phase II detoxification enzymes and may possess anticancer activity (*41*, *42*). The possibility that this Maillard-derived antioxidant compound is responsible for the decrease in PC-3 cell proliferation is currently under investigation.

Soy bread extracts (fraction 1S, 2S, and 3S) increased PC-3 cell proliferation at low concentrations. This stimulating effect on cell growth was reduced at higher concentrations of extracts. Soy bread crust (fraction 2S) was the only extract tested that significantly reduced PC-3 cell proliferation. Wheat bread extracts (fraction 1W, 2W, and 3W) increased PC-3 cell proliferation at all concentrations tested (results from fraction 1W and 2W were similar to fraction 3W, and therefore were not shown in **Figure 3**). The proposed mechanisms by which soy isoflavones may reduce cancer cell proliferation (*43*−*47*) include antioxidant activity of isoflavones (*48*), alteration of cell cycle distribution and induction of G2/M cell cycle arrest (*49*, *50*), induction of detoxification enzymes (*51*), and regulation of host immune function (*44*, *45*). Additional studies are needed to understand the mechanisms by which soy isoflavones delivered in a food matrix alter cancer cell proliferation and whether these mechanisms differ from those of pure isoflavones.

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